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COMPARATIVE CYTOGENETIC ANALYSIS OF THREE STYLOMMATOPHORAN SLUGS (MOLLUSCA, PULMONATA)

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INTRODUCTION

The Stylommatophora include 20,000 to 30,000 extant species of land snails and slugs, belonging to 70–90 families. Their classification system is still controversial because phylogeny and systematic relationships at the family level are poorly understood. Besides morphological studies, ribosomal RNA genes and the H3/H4 histone gene cluster (Ambruster et al., 2005; Wade et al., 2006) have also been used to resolve the relationships within this order. Recently, by comparison of primary sequence of mitochondrial and nuclear genes, Grande et al. (2004) resolved the Stylommatophora as the early split monophyletic sister group of all the other gastropod taxa.

Available data on the cytogenetics of Stylommatophora are extremely poor (reviewed by Patterson, 1969, and Thiriot-Quièvreux, 2003) and mostly concern the haploid (n) and/or diploid (2n) chromosome numbers and karyotype morphology. Vinogradov (2000) determined genome size (GS) and genome base composition in 15 pulmonate species, with five being stylommatophorans. In the last few years, only two thorough cytogenetic investigations have been carried out on *Milax nigricans*, *Cantareus aspersus*, and *C. mazzullii* (Vitturi et al., 2004, 2005), and their results proved to be useful to examine: (1) genomic organization at molecular level using fluorescent *in situ* hybridization (FISH) with repetitive DNA sequences as probes; (2) reciprocal position of ribosomal (18S–28S and 5S rDNA) and telomeric $(TTAGGG)_n$ multigene families using simultaneous double-colour FISH; and 3) evolution of genome by comparison of genome size (C-value) and base-pair composition.

In the present paper, we describe a cytogenetic comparative analysis of *Limacus flavus* (Linnaeus, 1758) (Limacidae), *Tandonia sowerbyi* (Férussac, 1823) (Milacidae), and *Deroceras panormitanum* (Lessona & Pollonera, 1882) (Agriolimacidae), carried out by standard (i.e., Giemsa and fluorochrome staining), and molecular (*in situ* hybridization) methods. Percentage of adenine-thymine DNA (AT-DNA) and genome size (GS) were also investigated for the three species and, in addition, for *Milax nigricans* (Philippi, 1836) (Milacidae), still unexplored on these parameters. This study was carried out with the aim of contributing to the karyological knowledge of the order Stylommatophora, hitherto broadly neglected from this point of view. In addition, we wanted to test whether cytogenetic analysis could reveal any inter-specific genomic differences that may be considered as informative characters in the phylogenetic tree construction.

MATERIAL AND METHODS

The specimens of *Limacus flavus* (Linnaeus, 1758) (N = 80), *Tandonia sowerbyi* (Férussac, 1823) (N = 50), *Deroceras panormitanum* (Lessona & Pollonera, 1882) (N = 55), and *Milax nigricans* (Philippi, 1836) (N = 30) were collected in the lawns around Polizzi Generosa, northwestern Sicily, Palermo Province, during 2004, 2005 and 2006. Sexually mature individuals occurred only on September–October (*L. flavus*, *T. sowerbyi*, and *M. nigricans*) or October–November (*D. panormitanum*) of each year. Specimens were identified according to the guidelines of Giusti (1973). Systematics follows the scheme proposed by Bodon et al. (1995). Vouchers of all investigated species were deposited in the laboratory of cytogenetics and molecular genetics (University of Urbino, Via Maggetti 22).

Chromosomes were obtained by air-drying from sexually mature testicular lobes after *in vivo* colchicine treatment (0.5 mg/ml for 1 h at room temperature), observed with a Leica microscope, and photographed with a Kodak Ektacolor 800 ASA film. Chromosome classifi-

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cation is according to Levan et al. (1964) and the centromeric index was evaluated using the method proposed by Naranjo et al. (1983). Metacentric, submetacentric and subtelocentric chromosomes (m, sm, st) were considered as bi-armed while acrocentrics and telocentrics (a, t) as mono-armed. In order to homogeneously compare karyotype formulas, subtelocentric chromosomes in *M. nigricans*, previously classified as mono-armed by Vitturi (1992), were re-considered herein as bi-armed. Slides were processed for conventional staining with Giemsa; whereas CMA_3 and DAPI were used for GC- and AT-specific fluorochrome banding, respectively (Sumner, 1990).

FISH was performed on fixed spermatocyte chromosomes using three different probes: a sea urchin (*Paracentrotus lividus*, Echinodermata) 18S rDNA (partial sequence) (Cantone et al., 1993); a $(TTAGGG)_n$ vertebrate-type hexamer repeat; and the $(GATA)_n$ microsatellite. The latter two probes were obtained by PCR in the absence of template (Ijdo et al., 1991) using $(TTAGGG)_5/(CCCTAA)_5$ and $(GATA)_7/(TATC)_7$ as primers, respectively. Nick translation labelling of the 18S rDNA probe was performed with digoxigenin according to manufacturer's instructions (Roche). Telomeric and $(GATA)_n$ probes were DIG-labelled following the random priming protocol (Roche).

Slides were pre-treated with RNAse A (200 µg/ml) and then chromosomes were denatured in 70% formamide/2xSSC for 4 min at 72°C. Probes were denatured for 5 min at 80 °C. Each slide was hybridized with 25 µl of the hybridization mixture containing 120 ng of labelled and denatured probe dissolved in hybridization solution (50% formamide, 10% dextran sulphate, 2xSSC, 50 mM sodium phosphate). Hybridization reaction was allowed to proceed overnight in a moist plastic chamber at 37°C. Slides were washed at 42°C, mounted in propidium iodide + antifade solution (2 µg/ml) and viewed under a Leica filter set I3 (BP 450–490, LP 515) which allowed the simultaneous visualization of fluorescein-labelled hybrid (yellow) and chromosomal (red) DNA.

Nuclear AT-DNA content and haploid DNA content (C-value) were evaluated through flow cytometric assay performed on cell suspensions obtained from deep frozen digestive gland of *L. flavus* (N = 7), *T. sowerbyi* (N = 12), *D. panormitanum* (N = 6), and *M. nigricans* (N = 10) specimens. Peripheral blood erythrocytes from chicken (2C GS = 2.52 pg, 2C AT DNA = 1.39 pg; Ronchetti et al., 1995) were added to the slug cell suspensions as internal standard. Methods for sample preparation and nuclear DNA content evaluation were the same described in Vitturi et al. (2005). A BRITE-HS cytometer (Bio-Rad Laboratories) equipped with a xenon-mercury lamp was used for the analyses. Data were reported as mean ± standard deviation.

RESULTS

Chromosome Analysis

Haploid chromosome number (30 metaphase-I plates analysed per species) resulted n = 31 in *L. flavus* (Fig. 1A) and *D. panormitanum* (Fig. 1B) and n = 33 in *T. sowerbyi* (Fig. 1C).

Karyotypes obtained from five Giemsa-stained spermatogonial metaphases per species appeared highly symmetrical in the three taxa showing all bi-armed chromosome pairs. In particular, *L. flavus* consists of 15 metacentric, 12 submetacentric, and 4 subtelocentric pairs (Fig. 2A); *D. panormitanum*, of 12 metacentric, 13 submetacentric, and six subtelocentric pairs (Fig. 2B); and *T. sowerbyi* of 21 metacentric, 9 submetacentric, and three subtelocentric pairs (Fig. 2C).

Fluorochrome application was uninformative for detecting any repeated and/or compartmentalised DNA sequences. In fact, counterstaining of spermatogonial metaphases with CMA_3 and DAPI failed to give positive or negative fluorescence showing all chromosomes as homogeneously stained (Figs. 3A, 4A). Similarly, C-banding did not provide visible C-positive blocks (data not shown). In contrast, the $(GATA)_n$ probe showed abundant hybridization sites, dispersed as dot-like fluorescent yellow signals throughout all chromosomes (Fig. 5A–C).

Ribosomal probe labelled (in yellow) the terminal region of one chromosome pair in *L. flavus* (Fig. 6A) and *T. sowerbyi* (Fig. 6C) whereas, in *D. panormitanum* (Fig. 6B) hybridised with the termini of five chromosome pairs.

In the three species, the $(TTAGGG)_n$ sequence revealed typical labelled areas (in yellow) at the ends of all chromosomes (Fig. 7A–C).

Genome Size and DNA Base Composition

Mean C-values of GS and AT-DNA percentage in the total genome are summarized in Table 1.

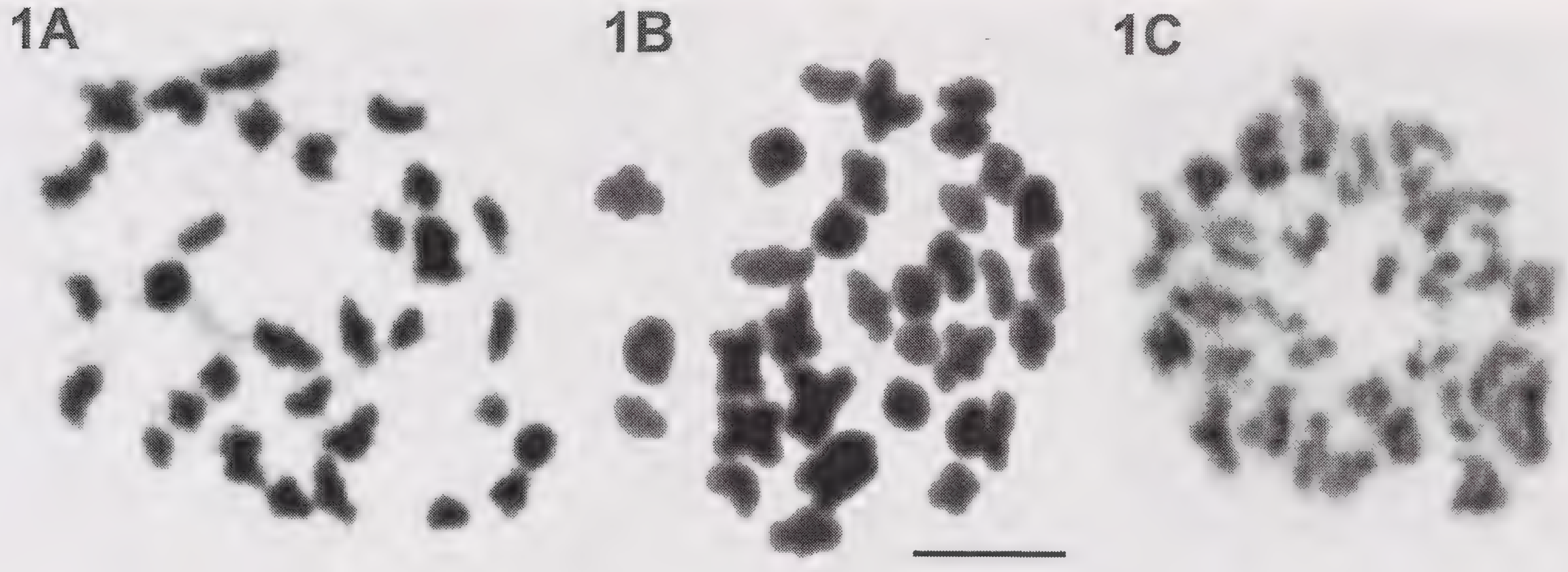


FIG. 1. Giemsa stained metaphase-I bivalents. 1A: *L. flavus*; 1B: *D. panormitanum*; 1C: *T. sowerbyi*. Scale bar = 10 µm.

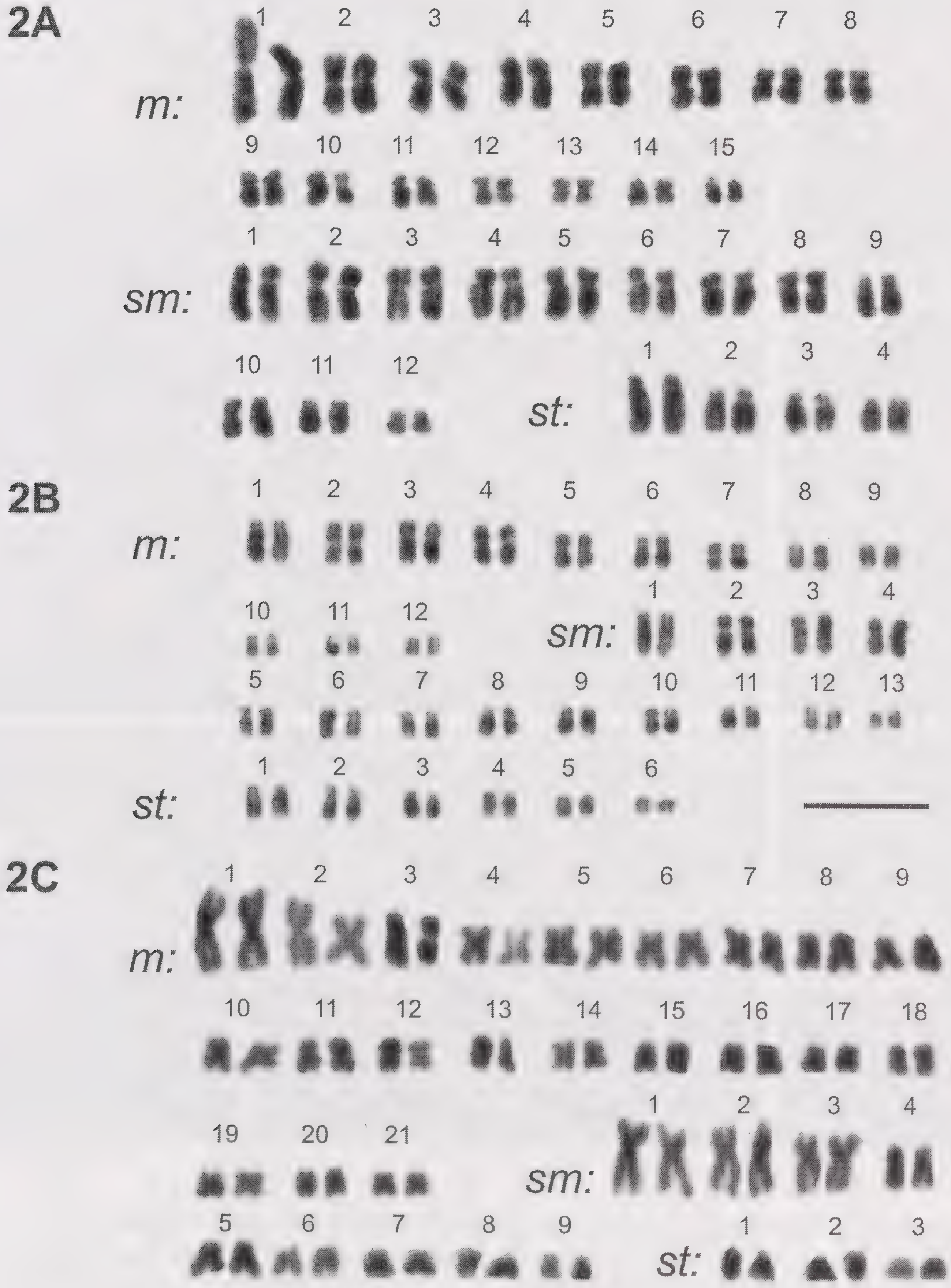
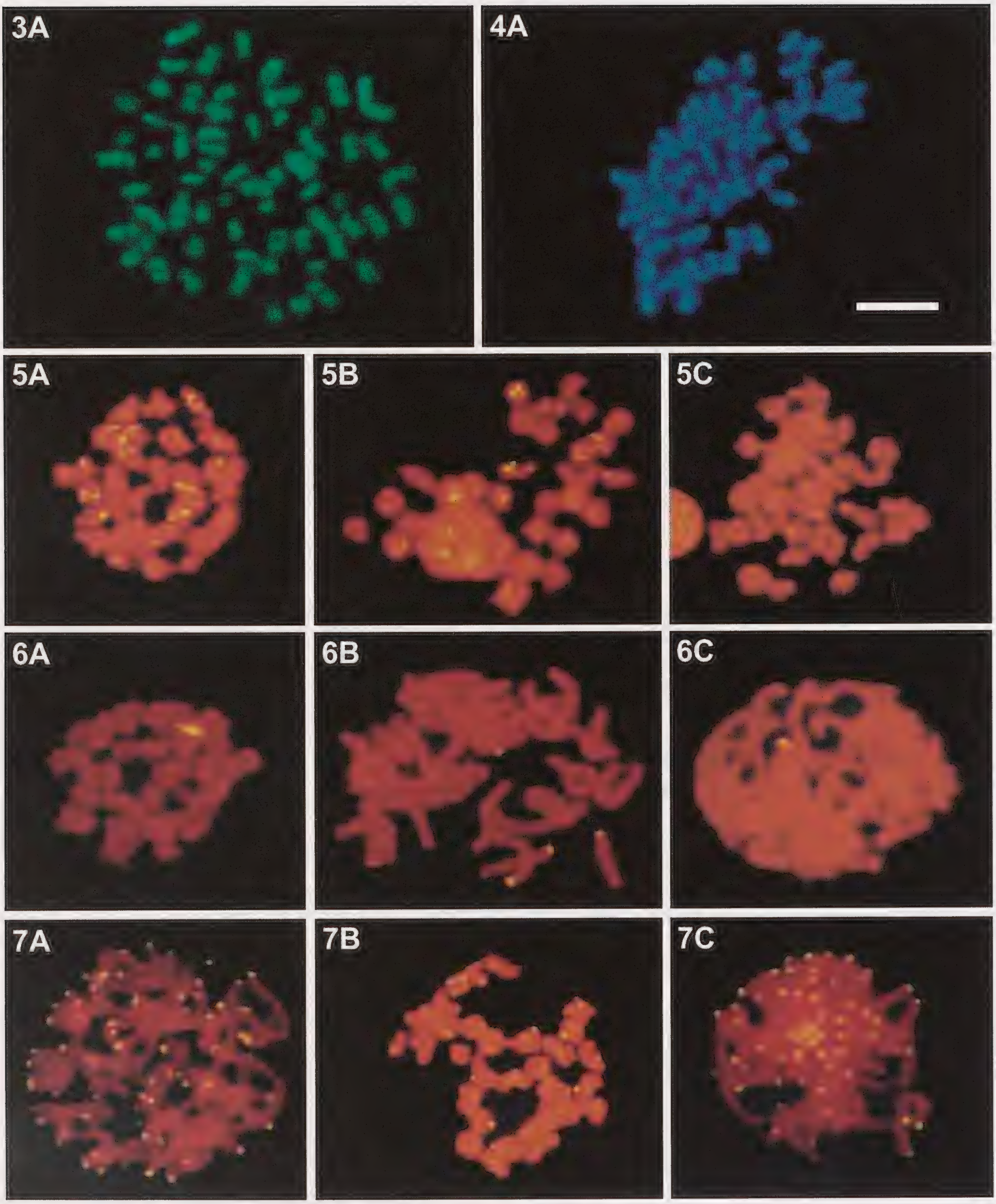


FIG. 2. Giemsa stained karyotype. 2A: *L. flavus* (2n = 62); 2B: *D. panormitanum* (2n = 62); 2C: *T. sowerbyi* (2n = 66). Chromosome pairs are shown clustered in three classes according to the chromosome morphology [metacentric (m), submetacentric (sm) and subtelocentric (st)] evaluated on the centromeric index. Scale bar = 10 µm.



FIGS. 3–7. Homogeneous staining patterns of spermatogonial metaphases of *L. flavus* with CMA$_3$ (green, 3A), and DAPI (blue, 4A) fluorochromes. Analogous dull fluorescence was observed in *D. panormitanum* and *T. sowerby* (data not shown); *in situ* hybridization patterns of the (GATA)$_n$ probe on spermatocyte chromosomes of *L. flavus* (5A); *D. panormitanum* (5B); and *T. sowerbyi* (5C); *in situ* hybridization with 18S rDNA (from *Paracentrotus lividus*) on spermatocyte chromosomes of *L. flavus* (6A); *D. panormitanum* (6B); and *T. sowerbyi* (6C). Yellow dots indicate where18S rDNA regions map on the chromosomes. (TTAGGG)$_n$ FISH patterns in *L. flavus* (7A); *D. panormitanum* (7B); and *T. sowerbyi* (7C). Scale bar = 10 μm.

TABLE 1. Compiled cytogenetical features in slug species of Milacidae, Limacidae, and Agriolimacidae (n.c. = not calculated).

Family	Species	n	2n	Karyotype formula	NOR number/ rDNA FISH	TTAGGG	Haploid DNA content (in pg) ± SD	AT %	References
Milacidae	*Milax gagates*	33 or 34					n.c.		Beeson, 1960
	Milax nigricans	33	66	32 (m + sm + st) + 1a			n.c.		Vitturi, 1992
	Milax nigricans	33	66		2	positive	n.c.		Vitturi et al., 2004
	Milax nigricans						2.34 ± 0.017	54.55	Present paper
	Tandonia sowerbyi	34					n.c.		Beeson, 1960
	Tandonia sowerbyi	33	66	21m + 9sm + 3st	2	positive	3.86 ± 0.049	53.85	Present paper
Limacidae	*Limax cinereoniger*	31					n.c.		Beeson, 1960
	Limax maximus	31					n.c.		Beeson, 1960
	Limacus flavus	31		15m + 12sm + 4st	2	positive	2.35 ± 0.014	53.43	Present paper
	Lehmannia marginata	24					n.c.		Beeson, 1960
	Lehmannia melitensis	20		20 (m+st)			n.c.		Vitturi & Sparacio, 1993
Agriolimacidae	*Deroceras agreste*	30					n.c.		Beeson, 1960
	Deroceras reticulatum	30					n.c.		Beeson, 1960
	Deroceras reticulatum						1.68	57.80	Vinogradov, 2000
	Deroceras panormitanum	31	62	12m + 13sm + 6st	10	positive	2.07 ± 0.014	49.17	Present paper

DISCUSSION

The three species show a substantial karyological homogeneity in (1) chromosome number and morphology; (2) fluorescent GC- or AT-specific staining patterns and (3) telomeric DNA composition. In fact, (1) their karyotypes share a haploid chromosome number (n = 31–33), which is common, although non exclusive, to the Limacidae, Agriolimacidae and Milacidae families (Patterson, 1969; Thiriot-Quievreux, 2003) and, as the majority of pulmonate species studied up to now, consist of all bi-armed elements; (2) both CMA_3 and DAPI dyes render all the chromosomes uniformly fluorescent; and (3) the hexamer $(TTAGGG)_n$ is the only motif observed in the telomeres.

Chromosome staining with Giemsa and fluorochromes were inadequate to reveal any inter-specific genomic difference, and the homogeneous DAPI and CMA_3 staining of chromatin indicated that GC- and AT-base pairs are equally interspersed throughout the genome. As previously reported for two Helicidae snail species of the genus *Cantareus* (Vitturi et al., 2005), CMA_3 was unable to identify the rDNA clusters. All the three species showed the $(TTAGGG)_n$ telomeric motif, as already reported for the other three investigated stylommatophoran species (Vitturi et al., 2004, 2005). This finding suggests that the "vertebrate" repeat $(TTAGGG)_n$ might be conserved within the order Stylommatophora. Conversely, a heterogeneous distribution of the vertebrate telomeric repeat has been already observed among Sacoglossa and Caenogastropoda since *Oxynoe olivacea* (Vitturi et al., 2000) and *Crepidula unguiformis* (Libertini et al., 2009) have a different telomeric motif although still unknown. Uniformity of Stylommatophora for the telomeric sequence can be clarified and resolved if more representatives species are analysed.

Karyological inter-specific heterogeneity becomes evident when the number of NOR sites and genome size (C-value) are compared. Major ribosomal sites are mapped at the terminal region in one chromosome pair (*L. flavus* and *T. sowerbyi*) or in five chromosome pairs (*D. panormitanum*). According to several authors, two NOR sites is presumed to be the plesiomorphic (ancestral) condition in vertebrates (Schmid et al., 1978) and invertebrates including molluscs (Pascoe et al., 1996; Thiriot-Quievreux, 2002; Vitturi et al., 2005). Taking into account this assumption, three out of four slug species analysed (Vitturi et al., 2004; present paper) seem to have retained the ancestral trait, whereas an increase in NOR number has occurred in *D. panormitanum,* probably through mechanisms of ribosomal cistrons translocation (Woznicki et al., 2000).

In spite of similarity in chromosome number, different genome sizes characterise the slugs of this study without showing any apparent evolutionary trend (Table 1). In fact, in *Tandonia sowerbyii* (Milacidae) GS amount (3.86 pg) is nearly the highest among Pulmonata (range 0.95–4.00 pg; Gregory, 2008), whereas in *D. panormitanum* (Agriolimacidae, 2.07 pg), *M. nigricans* (Milacidae, 2.34 pg), and *L. flavus* (Limacidae, 2.35 pg) GS values are similar and intermediate in the subclass range.

Observed inter-specific differences in GS values may reflect variations in highly and/or moderately repeated DNA quantities (Gregory, 2005). This assumption is not confirmed by C-banding which, in this case, failed to give any direct indication of constitutive heterochromatin. But, on the other hand, the presence of a high amount of $(GATA)_n$ DNA, would suggest that GS variation might depend on GATA-like short repeated sequences. A study on DNA contents in pulmonate gastropods (Vinogradov, 2000) reported on significantly higher GS and GC % (greater than 40%, i.e., corresponding to an AT-DNA percentage less than 60%) of terrestrial species as compared to related aquatic ones, suggesting as the most probable evolutionary trend a DNA accumulation accompanied by preferential increase in GC-fractions. Our data on GS and DNA base composition (Vitturi et al., 2005; present paper) confirm this tendency, which is consistent with Vinogradov's assumption that high GS and GC% may be considered as an adaptive trait for terrestrial life conferring a greater physical stability to DNA by the triple H-bond of guanine-cytosine and by dispersion of this base pair in the genome.

In conclusion, present results are certainly an extension of cytogenetic data in pulmonates, but the characters found were not informative on phylogeny of Stylommatophora, being either symplesiomorphies – chromosome morphology and number, genome base pair composition; the occurrence of the $(TTAGGG)_n$ telomeric motif; two NORs – or autoapomorphies – five NOR-bearing chromosome pairs (*D. panormitanum*); a high GS value (*T. sowerbyi*). However, the potential of cytogenetic characters for phylogenetic reconstruction may be clarified and resolved if more representatives of each family are included in the analyses and, above all, if reliable synapomorphies are discovered.

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LITERATURE CITED

ARMBRUSTER, G. F. J., M. BÖHME, D. BERNHARD & M. SCHLEGEL, 2005, The H3/H4 histone gene cluster of land snails (Gastropoda: Stylommatophora): TS/TV ratio, GC3 drive and signals in stylommatophoran phylogeny. *Journal of Molluscan Studies*, 71: 339–348.

BEESON, G. E., 1960, Chromosome numbers of slugs. *Nature*, 186: 257–258.

BODON, M., L. FAVILLI, F. GIUSTI & G. MANGANELLI, 1995, Gastropoda Pulmonata, in: A. MINELLI, S. RUFFO & S. LA POSTA, eds., *Checklist delle specie della fauna Italiana*, 16. Calderini, Bologna. 60 pp.

CANTONE, M., R. BARBIERI, G. DURO & G. GIUDICE, 1993, Sequence analysis of the rDNA spacer of *Paracentrotus lividus* and observations about pre-rRNA processing NTS sequence of *Paracentrotus lividus* rDNA. *Molecular Biology Report*, 18: 177–182.

GIUSTI, F., 1973, Notulae Malacologicae XVIII. I Molluschi terrestri e salmastri delle Isole Eolie. *Lavori della Società Italiana di Biogeografia*, (n.s.), 3: 113–306.

GRANDE, C., J. TEMPLADO, J. L. CERVERA & R. ZARDOYA, 2004, Molecular phylogeny of Euthyneura (Mollusca: Gastropoda). *Molecular Biology and Evolution*, 21(2): 303–313.

GREGORY, T. R., 2005, Genome size evolution in animals. Pp. 3–87, in: T. R. GREGORY, ed., *The evolution of the genome*. Elsevier, San Diego. xxvi + 740 pp.

GREGORY, T. R., 2008, Animal genome size database. URL http://www.genomesize.com

IJDO, J. W., R. A. WELLS, A. BALDINI & S. T. REEDERS, 1991, Improved telomere detection using a telomere repeat probe (TTAGGG)$_n$ generated by PCR. *Nucleic Acid Research*, 19: 4780.

LEVAN, A. R., K. FREDGA & A. A. SANDBERG, 1964, Nomenclature for centromeric position on chromosomes. *Hereditas*, 52: 201–220.

LIBERTINI, A., R. VITTURI, A. GREGORINI & M. COLOMBA, 2009, Karyotypes, banding patterns and nuclear DNA content in *Crepidula unguiformis* Lamarck, 1822 and *Naticarius stercusmuscarum* (Gmelin, 1791) (Mollusca, Caenogastropoda). *Malacologia*, 51: 111–118.

NARANJO, C. A., L. POGGIO & P. E. BRANDHAM, 1983, A practical method of chromosome classification on the basis of centromere position. *Genetica*, 62: 51–53.

PASCOE, P. L., S. J. PATTON, R. CRITCHER & D. R. DIXON, 1996, Robertsonian polymorphism in the marine gastropod *Nucella lapillus*: advances in karyology using rDNA loci and NORs. *Chromosoma*, 104: 455–460.

PATTERSON, C. M., 1969, Chromosomes of molluscs. *Proceedings of Symposia on Mollusca* (Marine Biology Association of India), 2: 635–686.

RONCHETTI, E., S. SALVADORI & A. M. DEIANA, 1995, Genome size and AT-content in Anguilliformes. *European Journal of Histochemistry*, 39: 259–264.

SUMNER, A. T.,1990, *Chromosome banding*. Unwin Hyman Ltd., London. xiv + 434 pp.

SCHMID, M., 1978, Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. *Chromosoma*, 66: 361–388.

THIRIOT-QUIÈVREUX, C., 2002, Review of the literature on bivalve cytogenetics in the last ten years. *Cahiers de Biologie Marine*, 43: 17–26.

THIRIOT-QUIÈVREUX, C., 2003, Advances in chromosomal studies of gastropod molluscs. *Journal of Molluscan Studies*, 69: 187–201.

VINOGRADOV, A. E., 2000, Larger genomes for molluskan land pioneers. *Genome*, 43: 211–212.

VITTURI, R., 1992, Conventionally stained chromosomes, constitutive heterochromatin and nuclear organizer regions in *Milax nigricans* (Gastropoda, Pulmonata). *Chromatin*, 1: 147–155.

VITTURI, R. & I. SPARACIO, 1993, Spermatocyte chromosome analysis of the slug *Lehmannia melitensis* (Lessona and Pollonera, 1891) (Mollusca, Pulmonata) using conventional, NOR- and C-banding techniques. *Caryologia*, 46: 189–199.

VITTURI, R., P. GIANGUZZA, M. S. COLOMBA, K. R. JENSEN & S. RIGGIO, 2000, Cytogenetics in the sacoglossan *Oxynoe olivacea* (Mollusca: Opisthobranchia): karyotype, chromosome banding and fluorescent *in situ* hybridization. *Marine Biology*, 137: 577–582.

VITTURI, R., L. SINEO, N. VOLPE, A. LANNINO & M. COLOMBA, 2004, Repetitive DNAs in the slug *Milax nigricans*: association of ribosomal (18S–28S and 5S) and (TTAGGG)$_n$ telomeric sequences in the slug *M. nigricans* (Mollusca: Gastropoda: Pulmonata). *Micron*, 35: 255–260.

VITTURI, R., A. LIBERTINI, L. SINEO, I. SPARACIO, A. LANNINO, A. GREGORINI & M. COLOMBA, 2005, Cytogenetics of the land snails *Cantareus aspersus* and *C. mazzullii* (Mollusca: Gastropoda: Pulmonata). *Micron*, 36: 351–357.

WADE, C. M., P. B. MORDAN & F. NAGGS, 2006, Evolutionary relationships among the pulmonate land snails and slugs (Pulmonata, Stylommatophora). *Biological Journal of the Linnean Society*, 87(4): 593–610.

WOZNICKI, P., L. SÁNCHEZ, P. MARTÍNEZ, B. G. PARDO & M. JANKUN, 2000, A population analysis of the structure and variability of NOR in *Salmo trutta* by Ag, CMA$_3$ and ISH. *Genetica*, 108: 113–118.

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